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Macroscopic Orientation of Natural and Model Membranes for Structural Studies

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One approach for obtaining high-resolution structural and functional information for biomembranes and their proteins is by static solid-state NMR of oriented systems. Here, a general procedure to align fully functional biological membranes containing large membrane proteins ($M_r > 30,000$) is described. The method, based on the isopotential spin-dry ultracentrifugation technique, relies on the centrifugation of membrane fragments onto a support with simultaneous, or subsequent, partial evaporation of the solvent which aids alignment. The quality of orientation, as shown by the mosaic spread of the samples, was monitored by static solid-state ^{31}P NMR for the phospholipids and by ^2H NMR for a deuterated retinal in bovine rhodopsin. The generality of this method is demonstrated with three different membranes containing bovine rhodopsin in reconstituted bilayers, natural membranes with the red cell anion exchange transport protein in erythrocytes, band 3, and the nicotinic acetylcholine receptor. © 1997 Academic Press

The elucidation of the structural and functional organization of biological membranes by various biophysical techniques is one of the important questions being addressed in structural biology. The complexity of the systems, however, their supramolecular structure, and their dynamic properties make direct studies particularly difficult. Specifically, membrane proteins present extraordinary technical difficulties to the most commonly used methods in structural biology, such as crystallography (1) and solution NMR spectroscopy (2). In

recent years solid-state NMR spectroscopy (3, 4) on macroscopically oriented lipid bilayers has rapidly emerged as an alternative approach to elucidate structural and functional features of membrane-bound peptides and proteins. In such aligned systems, lipids and proteins are arranged uniaxially around the membrane, allowing normal orientation of the molecule backbone relative to the substratum. In combination with isotopic labeling (e.g., ^2H , ^{13}C , ^{15}N) this NMR approach has successfully been used to determine the complete secondary structure of the M2 channel peptide and fd coat protein (3), while the orientation of the antibiotic peptide magainin has been resolved in bilayers (5). The complete secondary structure of gramicidin and its dynamic properties in membranes have also been obtained using ^2H and ^{15}N NMR (6–9). In addition, the complete structure and orientation of deuterated retinal in bacteriorhodopsin at different states of its photocycle has been resolved (10). The average molecular and orientational order of lipids and peptides in liquid crystalline membranes could also be determined unambiguously in this way (11–14).

Unfortunately, the generation of structural and orientational information about membrane proteins has been limited mainly to peptides, since no general methods have been available for aligning native and reconstituted membranes containing larger integral membrane proteins in sufficient quality and quantity without affecting their structural integrity or function. Previous alignment techniques include practical drawbacks, such as pressure annealing (15), rehydrating and temperature annealing (16, 17), smearing of membrane suspension on plates with partial dehydration (16, 17), or require magnetic orientation of phospholipid systems (18, 19) or natively stacked membranes (20).

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The recent development of the isopotential spin-dry ultracentrifugation technique (ISDU)² (15, 21, 22) is, on the other hand, capable of preserving biological integrity while still producing good membrane alignment. Here, we describe a general procedure for the macroscopic orientation of biological membranes in order to perform structural, functional, and dynamic studies on the various membrane components, mainly larger membrane proteins ($M_r > 30,000$), specifically by solid-state NMR. The versatility of the ISDU technique is demonstrated with various membranes containing proteins from completely different families, namely the 7-TM GPC-receptor bovine rhodopsin ($M_r \sim 39$ K), the anion exchange transport protein band 3 ($M_r \sim 85$ – 100 K), and the nicotinic acetylcholine receptor ($M_r \sim 280$ K). Our results, obtained by probing the quality of lipid alignment (as an upper limit for the membrane protein alignment) by ³¹P NMR, suggest that this approach not only is suitable for solid-state NMR studies because we could already demonstrate by ²H NMR on selectively deuterated rhodopsin, but also can be used generally for various diffraction and other spectroscopic methods (15, 22–26).

MATERIALS AND METHODS

Isopotential Spin-Dry Ultracentrifugation Technique

The isopotential ultracentrifuge cell was originally designed by Clark and Rothschild (21, 22) and later modified by Freed *et al.* (15). For our purpose the cell was built for use in a SW 28 rotor (Beckman). The cell reservoir body, support plug, and fill insert for the bottom of the tube were made of polycarbonate which, in tests, showed higher resistance to deformation at high rotor speeds compared to Delrin. Stainless steel was used for the three-dimensional isopotential surface plate. All other details were used as described elsewhere (15). The evaporation rate was controlled by screw caps with different pin hole sizes (50–1000 μ m). For the preparation of membrane samples without dehydration, sealed caps were used.

Sample Preparation

Oriented samples were prepared on grease-free Melinex (Agar Scientific, Stansted, UK) or 73- μ m-thin glass plates (Marienfeld Glassware, Bad-Mergentheim, Germany) previously cleaned in hot nitric acid and rinsed extensively in double-distilled water. The isopotential cell was filled with membrane suspension and centrifuged (40,000–90,000g; 4°C; for up to 18 h) with and

without simultaneous evaporation of water. The pelleted membrane samples were kept at controlled humidity and temperature. For NMR experiments, typically two plates were stacked together with spacers, each containing up to 4 mg of total membrane. Afterward the samples were placed in either 8- or 10-mm sealed NMR tubes with 30 μ l of appropriate salt solution for controlled humidity.

Bovine Rhodopsin in Native and Reconstituted Membranes

Bovine rod outer segments (ROS) were prepared as described (27, 28). Briefly, ROS were isolated and purified by sucrose density centrifugation to yield intact photoreceptor disks without peripheral proteins. These disk membranes were used directly for the ISDU. In some experiments, reconstituted membranes were prepared by purification of the protein from photoreceptor disk membranes (27, 28) and then incorporated into DMPC (Sigma) bilayers at lipid:protein molar ratios of 40:1 and 100:1, respectively. Finally, 450 μ l ROS membrane suspension containing 5 nmol of rhodopsin were centrifuged (40,000g, 4°C, 12 h) under simultaneous evaporation. For reconstituted DMPC/rhodopsin complexes, a 500- μ l suspension in water was used which contained 6 nmol of rhodopsin for a 100:1 molar ratio complex and 10 nmol of rhodopsin for 40:1 molar ratio complex, respectively. Afterward the membrane films were rehydrated (52% humidity, 4°C, 48 h). For ²H NMR experiments rhodopsin in ROS membranes was regenerated (29) with 11-*cis*-retinal, which was specifically deuterated on the methyl group at the C₂₀ position. After purification of the protein DMPC/rhodopsin complexes were prepared at a lipid:protein molar ratios of 50:1 as described above. Finally, 900 μ l membrane suspension in deuterium-depleted water (Aldrich, UK) containing 20 nmol of rhodopsin were centrifuged (80,000g, 4°C, 12 h) on glass plates under simultaneous evaporation and membrane samples kept at controlled humidity (52%, 4°C). For NMR experiments 40 plates containing aligned membranes were placed in a 10-mm round NMR sample tube and finally sealed.

Erythrocyte Membranes Containing Band 3

Hemoglobin-free erythrocyte ghosts were prepared from recently outdated human blood by hemolysis and repeated washing in 10 vol of ice-cold 5 mM phosphate, 0.5 mM EDTA, 20 μ g/ml PMSF, pH 8 (30). The final wash was in 10 mM Tris/10 mM NaCl/0.5 mM EDTA/2 mM β -mercaptoethanol, pH 8. Orientation of the erythrocyte membrane was adapted from the method of (31) with 1 ml of ghost membrane (4 mg/ml protein, as determined by a modified Lowry assay) pelleted onto a Melinex sheet by centrifugation (90,000g, 4°C, 16–

² Abbreviations used: ISDU, isopotential spin-dry ultracentrifugation; ROS, rod outer segments; DMPC, dimyristoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CSA, chemical shift anisotropy; nAChR, nicotinic acetylcholine receptor; PMSF, phenylmethylsulfonyl fluoride.

20 h) without drying. The membrane film was incubated (81% humidity, 20°C, for 4–6 h) to effect partial dehydration to improve membrane orientation, as determined by ^{31}P NMR. Samples for electron microscopy were fixed in 1% osmium tetroxide solution and processed by standard procedures for thin sectioning (31).

nAcChoR-Rich Membranes

nAcChoR-rich membranes isolated from the *Torpedo nobiliana* were prepared by sucrose density centrifugation (32). Following isolation membranes were suspended in 20 mM EDTA buffer (pH 7.2) for ISDU. Orientation was achieved by pelleting 1 ml of buffer containing 1 mg *nAcChoR* membranes onto Melinex by centrifugation (90,000g, 4°C, 15 h) without drying. Samples were then removed and partially dried (for another 2 h, 20°C, 81% relative humidity) to improve further the membrane orientation.

NMR Measurements

Phosphorus NMR. The phospholipid ^{31}P chemical shift anisotropy (CSA) in static solid-state NMR spectra (33) is a very sensitive method for assessing the degree of alignment of phospholipids in bilayers. The CSA tensor is axially symmetric for phospholipids undergoing fast rotation around an axis perpendicular to the bilayer plane and the principal values are σ_{\perp} and σ_{\parallel} with the CSA anisotropy usually expressed as $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$. For random, unoriented multilamellar bilayer dispersion, a broad spherically averaged powder spectrum is obtained whose full spectral width is described by $\Delta\sigma$. For a specific phospholipid in an oriented bilayer in the L_{α} phase, the ^{31}P NMR spectrum consists of a single resonance line whose frequency ν_{CSA} increases with the angle θ between the membrane normal and the magnetic field according to $\nu_{\text{CSA}}(\theta) = (2/3 \Delta\sigma)(3\cos^2\theta - 1)$.

NMR measurements were performed on a Bruker MSL 400 spectrometer at 161 MHz for ^{31}P using a spin 1/2 Hahn-echo pulse sequence (34) and broadband ^1H decoupling (25 kHz) with appropriate phase cycling. The pulse spacing was 50 μs and the width of the 90° pulses were 5 μs for an 8-mm coil and 6 μs for a 10-mm coil. Typically 500–2000 scans were accumulated with a 4-s repetition time. The temperature of the samples was regulated to within $\pm 1^\circ\text{C}$ by a control unit (Bruker VT 1000). All chemical shifts were assigned relative to external 85% H_3PO_4 . The quality of orientation is expressed in a value for the mosaic spread and was assessed by analyzing the shape and width of the ^{31}P NMR resonances using a lineshape simulation algorithm based on described methods (10, 35).

Deuterium NMR. Orientation-dependent ^2H NMR spectra of aligned, rhodopsin-containing DMPC mem-

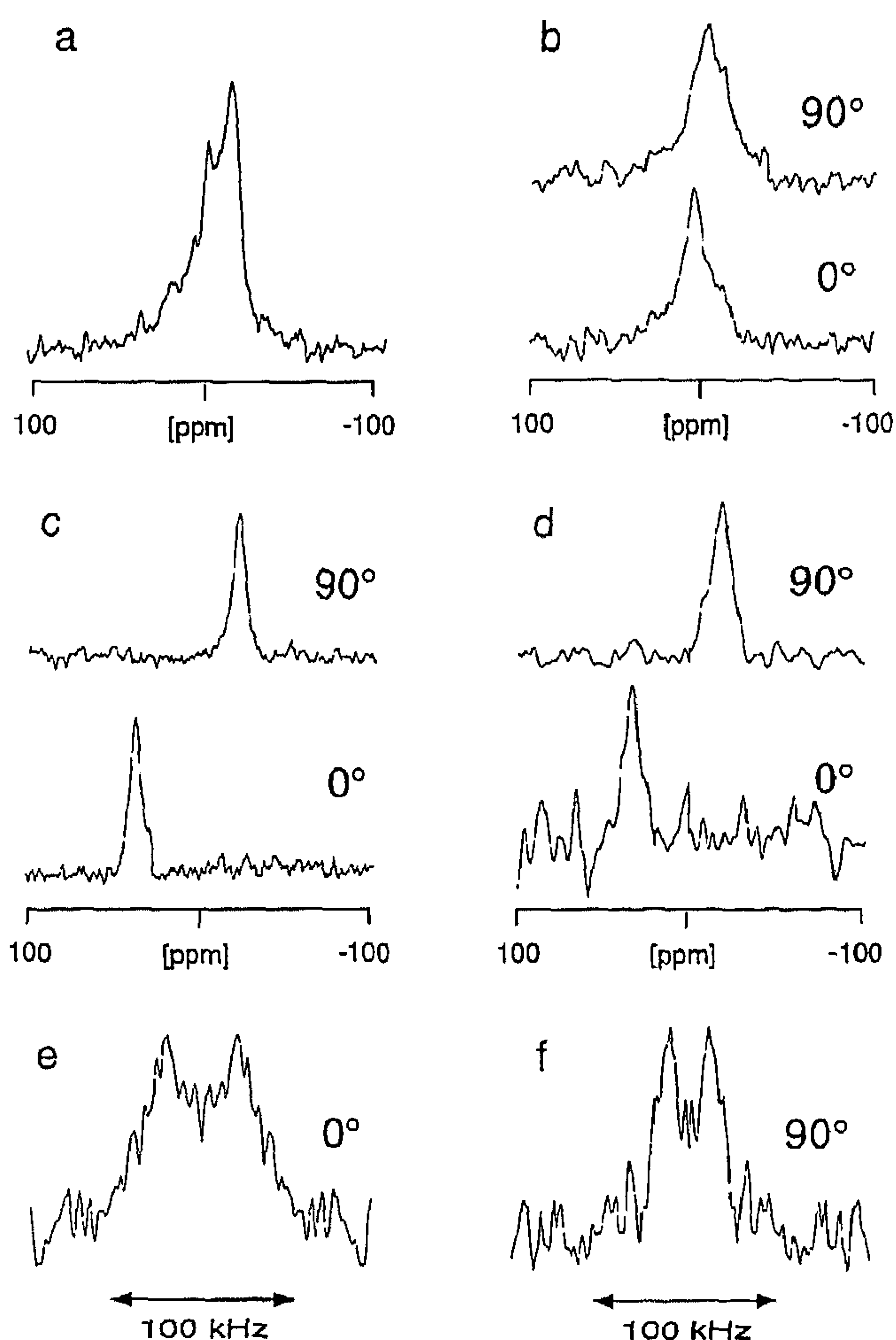


FIG. 1. Phosphorus-31 NMR spectra (161 MHz) of natural and reconstituted membranes containing bovine rhodopsin at RT as unoriented fully hydrated ROS disk membranes (a), aligned natural ROS disk membranes (b), for aligned reconstituted DMPC/rhodopsin bilayers at a 100:1 lipid:protein molar ratio (c), as in c but at a lipid:rhodopsin molar ratio of 40:1 (d). Deuterium NMR spectra (61 MHz) at 200K of aligned DMPC/rhodopsin membranes at 50:1 lipid:protein molar ratio (e) and (f). All spectra in b–f were measured with the field direction either parallel (0°) or perpendicular (90°) to the membrane normal. See text for experimental details.

branes were acquired at 61 MHz using a quadrupolar echo pulse sequence (90°- τ -90°) with a 5.5- μs -long 90° pulse, an interpulse delay of 30 μs , a recycle time of 400 μs , 100,000–150,000 scans, and appropriate standard quadrature-phase cycling.

RESULTS AND DISCUSSION

Native ROS Disk Membranes

Phosphorus-31 spectra of unoriented and aligned ROS disk membranes tilted at different angles relative to the applied magnetic field are shown in Figs. 1a and 1b. For unoriented ROS disks, an axially symmetric powder spectrum (Fig. 1a) was observed, typical for

multilamellar vesicles where the lipid molecules are randomly distributed with respect to the magnetic field. The reduced CSA obtained from the outer edges of the powder pattern is 42 ppm, a value characteristic for fully hydrated ROS disks and other PC/PE-rich membranes in the liquid crystalline phase (36–38). In Fig. 1b, spectra of aligned ROS disk membranes prepared by ISDU at 0° and 90° orientation relative to the applied field are shown. The observed ^{31}P NMR lineshape at 0° clearly reveals a superimposition of two components, indicated by a broad powder-like spectrum between 35 and –20 ppm, and a large central resonance at 3.9 ppm. In the 90° spectrum, the resonance of the central component shifts to –2 ppm, while the powder-like component remains similar with some additional intensity at the right edge. The absence of a resonance line at 28 ppm for the 0° orientation and at –14 ppm for the 90° orientation with respect to the applied field indicates that the sample does not contain uniaxially aligned bilayers in the L_α phase. The observed lineshape and its orientational dependence (Fig. 1b) suggest that the lipid molecules were arranged in either nonoriented bilayers, reflected in the powder subspectrum, or nonbilayer isotropic phases, resulting in a central resonance. However, the lineshape of this overlapping central peak and its small angular dependence of 6 ppm does not allow differentiation between hexagonal, cubic, or other nonbilayer phases. X-ray and electron microscopy studies on ROS disks aligned by the same technique assumed a binary-phase composition of concentrated protein containing lamellae interrupted by hexagonally packed tubes of lipids in H_{II} phase and clustered particles (25). Our results support their suggestion. Surprisingly, optical linear dichroism (22) and polarized Fourier transform infrared spectroscopy (39) showed that rhodopsin itself is highly ordered in these membranes and is still functionally intact. In contrast to X-ray and ^{31}P NMR methods, these optical techniques directly probe the orientation of the protein, whereas X-ray and ^{31}P NMR techniques determine the orientation of the lipids.

Reconstituted DMPC/Rhodopsin Membranes

Since the quality of lipid alignment in native ROS disks membranes as described above was not high, rhodopsin was reconstituted into DMPC bilayers and alignment was performed by applying the ISDU technique (other methods failed, spectra not shown). The spectra for aligned DMPC bilayers containing bovine rhodopsin at different concentrations are shown in Fig. 1c (100:1, lipid:protein molar ratio) and Fig. 1d (40:1, lipid:protein molar ratio). The spectra consist of a single line which varies as a function of the sample inclination from 40 ppm at 0° to –20 ppm at 90° orientation. This angular dependence is in excellent agreement with the prediction for oriented bilayers where the

phospholipid molecules undergo fast motions around the membrane normal (33). No unoriented material was detected in the spectrum. The angular dependence of NMR lineshapes and the observed CSA value of 60 ppm clearly showed that DMPC/rhodopsin membrane at both 100:1 and 40:1 (lipid:protein molar ratio) form stable, uniaxially oriented bilayers in the liquid-crystalline phase (40). The measured 60 ppm for $\Delta\sigma$ was slightly higher than for pure, fully hydrated DMPC bilayers (40) due to a lower hydration (52% humidity) in the samples measured here. The observed linewidths at 0° orientation of 8 ppm for 100:1 (lipid:protein molar ratio) complex and 12 ppm for 40:1 (lipid:protein molar ratio) complex compared to 2 ppm for pure DMPC membranes (spectra not shown) were caused mainly by a distribution of membrane directors reflecting the mosaic spread, and therefore the quality of the samples. It is suggested that the protein possibly prevents a better bilayer lipid alignment (41). By decreasing the lipid-to-protein ratio in the complex a greater distribution of membrane directors is achieved around the alignment axis, which was shown by the linewidth values obtained for the mosaic spread and increases from about $13 \pm 2^\circ$ for the 100:1 (lipid:protein molar ratio) complex to about $22 \pm 2^\circ$ for the 40:1 (lipid:protein molar ratio) complex. The intensity of the ^{31}P NMR signal decreases with decreasing lipid-to-protein ratio since the total amount of aligned membranes on two plates was kept constant. The NMR spectrum obtained was sufficient to analyze the alignment of the sample using only a small amount ($<0.8 \mu\text{g}$) of protein in the membranes. The results obtained indicate that DMPC is suitable for preparing uniaxially oriented membrane samples for structural and functional studies on integral membrane proteins, such as rhodopsin, for cases where this is not possible for natural membrane suspensions. As an example, ^2H NMR spectra were acquired for rhodopsin in aligned DMPC bilayers, where rhodopsin was deuterated at the C_{20} methyl group of its chromophore, 11-*cis*-retinal. The spectra obtained for rhodopsin at the dark-adapted state at 200 K for the 0° and 90° inclination are shown in Figs. 1e and 1f. At 0° orientation the lineshape exhibits a quadrupolar splitting of 41 kHz which is reduced to 22 kHz at 90° orientation. The orientation dependence of the lineshapes was then directly used to determine the C—CD₃ bond angle in the protein, which was obtained to $30 \pm 5^\circ$ with respect to the protein long axis, which is collinear with the membrane normal.

Erythrocyte Membranes

Phosphorus-31 NMR spectra for unoriented erythrocyte membrane dispersions and aligned erythrocyte membranes are shown in Figs. 2a and Fig. 2b, respectively. The spectrum for unoriented ghost membranes (Fig. 2a) shows a powder pattern with a superimposed

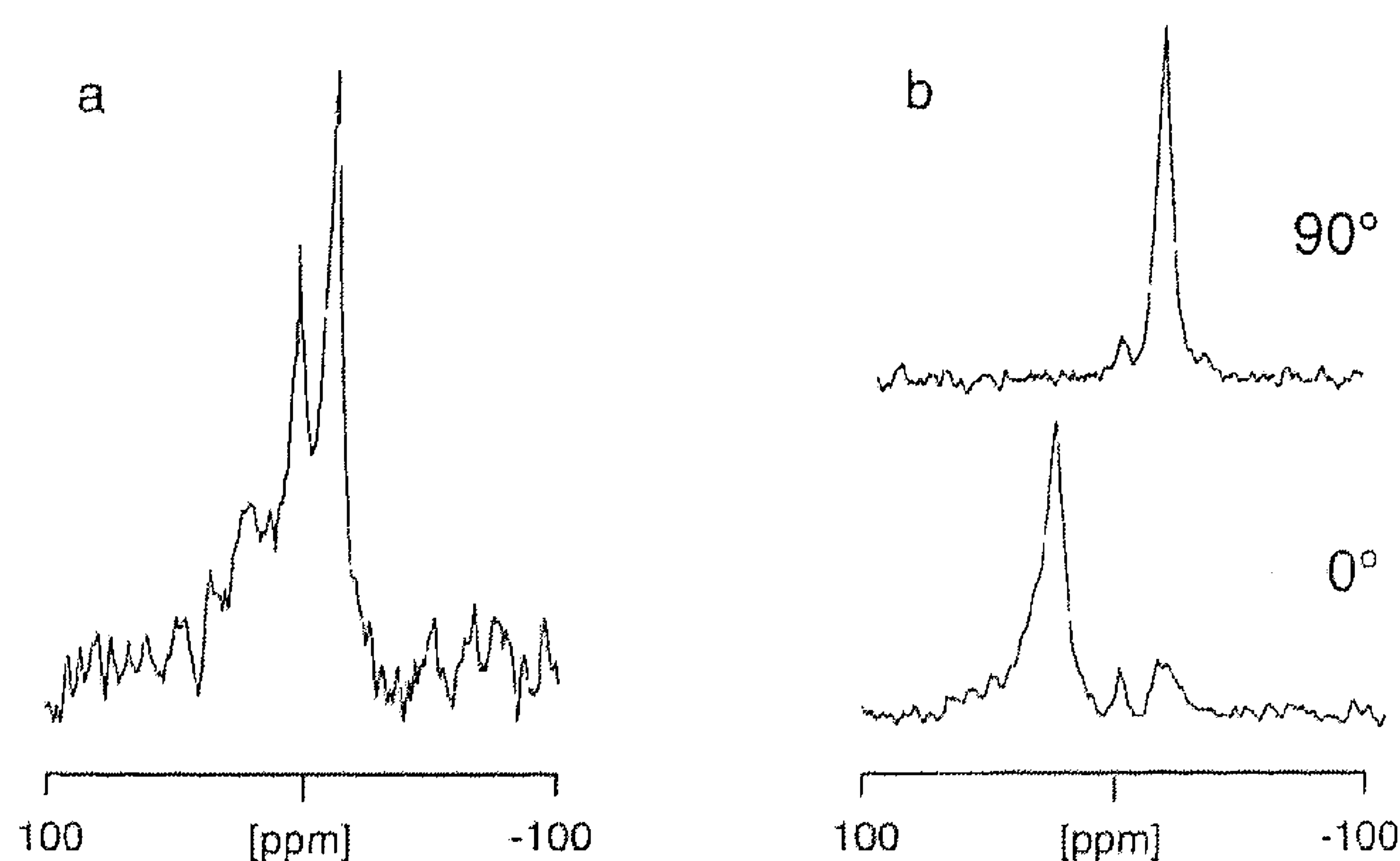


FIG. 2. Phosphorus-31 NMR spectra of fully hydrated unoriented (a) and aligned (b) native erythrocyte membranes at RT. Spectra in b were measured with the field direction either parallel (lower) or perpendicular (upper) to the membrane normal. See text for experimental details.

sharp central line at -2 ppm. The broad lineshape has a low-field shoulder at 28 ppm and a high-field shoulder at -15 ppm, demonstrating the axial symmetry of the ^{31}P nuclei, indicating that the lipids are in the liquid-crystalline state, which confirms earlier results (42). The central resonance is probably caused by a fraction of lipids which undergo isotropic motions, either in small vesicles or because of strong specific protein-lipid interactions.

The spectrum of aligned erythrocyte membranes at the 0° inclination with respect to the applied field presented in Fig. 2b shows a large single resonance at 30 ppm which is shifted to -14 ppm when the membranes are at 90° orientation (Fig. 2b, top), as expected for aligned membranes. About 10% of the membrane was not oriented, causing a powder-like spectrum at -15 ppm in the 0° spectrum. A spectral line at 0 ppm can also be detected in this spectrum which accounts for ca. 3% of lipids, probably in a form of small lipid particles, characteristic of natural membranes (43). The quality of alignment could be determined by analyzing the observed 8 ppm linewidth for the main ^{31}P phospholipid resonance in the 0° spectrum. A careful simulation of the lineshapes, including the different known CSA values for the PC and PE lipids, yielded to a value of $20 \pm 2^\circ$ for the mosaic spread of the membranes.

Despite the heterogeneous lipid composition of the membrane, separate ^{31}P signals from PE and PC lipids were not resolved and an average CSA value of 44 ppm was observed from the 0° and 90° spectra (Fig. 2b) which is in good agreement with the 42 ppm observed in unoriented erythrocytes (Fig. 2a) and previous studies (42). CSA values of the powder and oriented sample are similar, implying that our alignment procedure produced sufficiently hydrated, oriented membranes near their biologically active state. The NMR results

obtained were supported by electron microscopy (Fig. 3) in which well aligned, parallel arranged bilayers are observed, occasionally interrupted by nonbilayer structures or unoriented bilayer fragments.

The results observed by us support our approach of partial dehydration followed the ISDU procedure, to improve the macroscopic alignment. To obtain optimal alignment the degree of improvement could easily be monitored at various humidities and time intervals using ^{31}P NMR, polarization microscopy, or EPR, where 3-doxyl-17 β -hydroxy 5 α -androsterane (Sigma) as a spin label enabled an easy detection of the macroscopic order. Once the time period and dehydration level were worked out, the method was routinely used without any problem, since the tolerance limits in humidity and time period are very high. For providing large stacks of aligned membranes for sensitive NMR experiments, we could also easily keep the membranes after pelleting them on glass plates for weeks at -20°C , followed by partial dehydration for all plates (about 40) only shortly before the experiment without any loss of quality of alignment compared to unfrozen samples.

Nicotinic Acetylcholine Receptor-Rich Membranes

The ^{31}P NMR spectra for aligned nAChR-rich membranes at 0° and 90° tilt angles with respect to the magnetic field are shown in Fig. 4. A shift of spectral intensity from approximately 30 ppm at 0° to -15 ppm at 90° is seen, indicating an homogeneous ordering of the membrane bilayers with respect to the membrane normal. At the 0° inclination of the membrane stacks



FIG. 3. Thin section electron micrograph of aligned erythrocyte membranes as used in the NMR experiments, sectioned perpendicular to the membrane plane (accelerating voltage 80 kV, original magnification $\times 50,000$). Samples were prepared by fixing in 1% osmium tetroxide, embedded in araldite, and sectioned to a thickness of 90 nm. Micrograph was taken on a Jeol 2000 EX. The bar represents 600 nm.

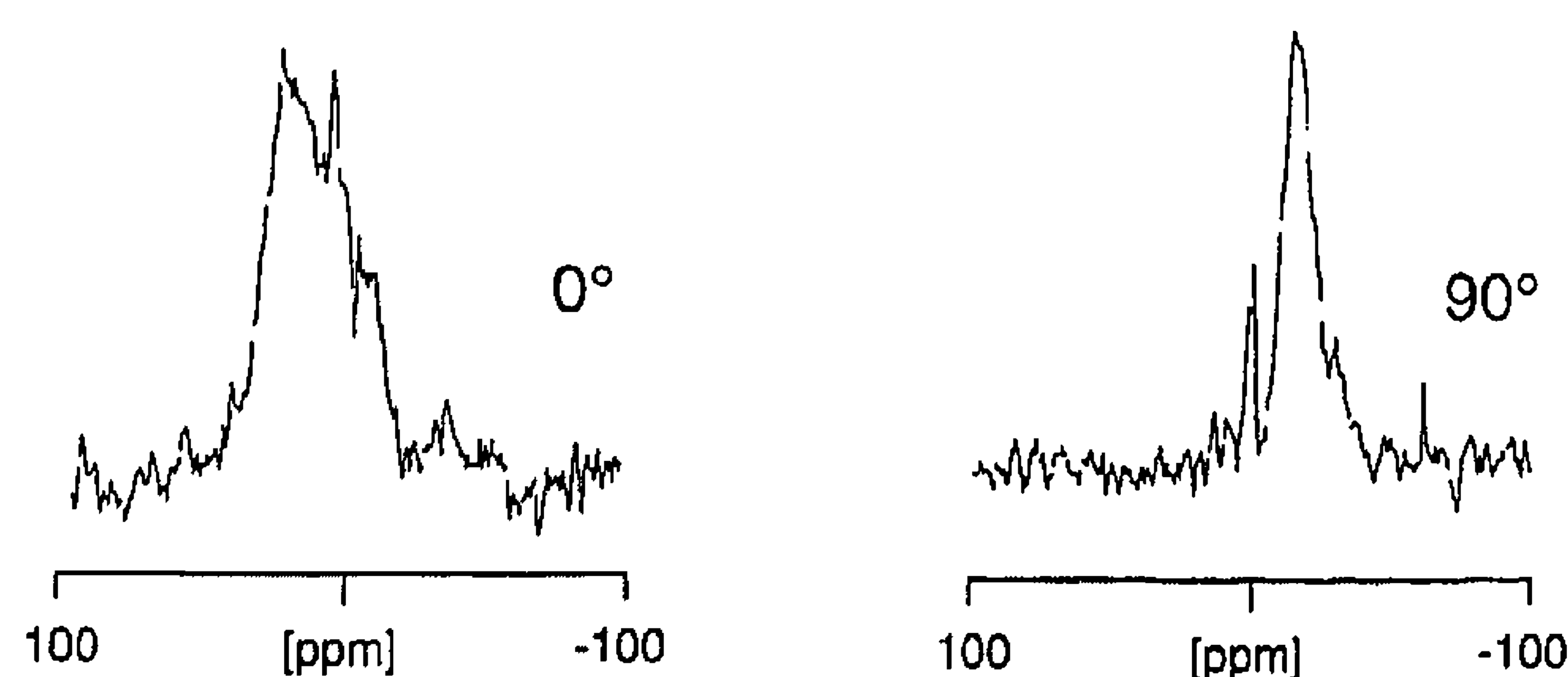


FIG. 4. Phosphorus-31 NMR spectra of aligned native nAcCho-rich membranes at RT. Spectra were measured with the field direction either parallel (left) or perpendicular (right) to the membrane normal. See text for experimental details.

with respect to the magnetic field, a broad lineshape covering a range from 30 to -10 ppm is observed with the main resonance at 26 ppm. The breadth of the spectrum arises from the mosaic spread of the membrane and the diverse chemical shift anisotropies of the lipid species present in membranes derived from *Torpedo* electric organs (44). The averaged reduced CSA anisotropy was measured as 45 ppm which is typical for PC/PE bilayers in the L_α phase (37, 42). The spectrum at 0° also exhibits a resonance line at -12 ppm (Fig. 4), indicating a small fraction of unoriented material, while the observed resonance at 0 ppm in the spectrum at 90° indicates either nonbilayer structures and small clustered particles or phosphorylated protein. The main resonance lines in both spectra (Fig. 4) were simulated to estimate the average mosaic spread, which was determined to be $30 \pm 3^\circ$, reflecting a slightly larger distribution of membrane lipid directors around the axis of orientation compared to the erythrocyte membranes. Nevertheless, the NMR results demonstrated that the partial dehydration approach, as worked out for the erythrocyte membranes, could also have easily been applied and used as a routine method for alignment of nAcChoR-rich membranes.

CONCLUSIONS

Static, solid-state NMR on oriented membrane systems (3–10) provides a useful method to resolve structural and functional details of large integral membrane proteins. In this approach, the NMR spectral anisotropy of appropriate NMR nuclei is determined to give direct molecular structure for specifically introduced nuclei, such as ^2H which shows quadrupolar anisotropy, or ^{13}C , ^{31}P , or ^{15}N which shows chemical shift anisotropy. For this approach to succeed, the membrane and its protein component, with associated labeled ligand where appropriate, must possess a reasonable degree of macroscopic orientation with respect to the applied field. Here, we have successfully developed a general alignment procedure, based on the ISDU

technique originally introduced by Clark and Rothschild (21, 22) to orient various membranes near their physiological state in sufficient amounts for NMR studies as confirmed by ^{31}P NMR and electron microscopy. By using static ^{31}P NMR probing the lipid alignment, the total amount of required membranes could be kept to a minimum, which is specifically important for studies on membrane-protein systems available only in limited quantities.

The generality of the approach is demonstrated on three important integral membrane proteins, namely bovine rhodopsin (a 7-TMD receptor), band 3 anion transporter, and the nicotinic acetylcholine receptor. Except for rhodopsin, the other systems could be oriented in their native membranes. By reconstituting rhodopsin into DMPC bilayers, conditions could be found where not only the protein but also the lipids were uniaxially arranged in a bilayer where the protein still possesses its functional activity (45). Static ^{31}P NMR was used as a highly sensitive method to characterize the degree of alignment and functional integrity of the bilayer lipids by probing the phospholipids (33, 42). Using an appropriate ^{31}P NMR lineshape analysis algorithm, the quality of alignment has been determined as a mosaic spread (10, 35), a parameter which reflects the deviation of membrane directors from the orientation axis. Although obtained from lipids, the aligned proteins can be assumed to have at least the same degree, or better, of mosaic spread (35, 46, 47). The validity of the approach on proteins could already have been successfully demonstrated by us on the integral membrane protein rhodopsin containing selectively deuterated retinal where we determined orientational constraints for the chromophore. The quality of oriented membranes obtained by our method is therefore sufficient for various structural solid-state NMR studies on membrane proteins, and additionally for other biophysical techniques.

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